Current Biology, Vol. 14, 2076–2085, December 14, 2004, ©2004 Elsevier Ltd. All rights reserved. DOI 10.1016/j.cub.2004.11.054

Suppression of Constant-Light-Induced Blindness but Not Retinal Degeneration by Inhibition of the Rhodopsin Degradation Pathway

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Summary

Background: Continuous exposure to light, even at relatively low intensities, leads to retinal damage and blindness in wild-type animals. However, the molecular mechanisms underlying constant-light-induced blindness are poorly understood. It has been presumed that the visual impairment resulting from long-term, continuous exposure to ambient light is a secondary consequence of the effects of light on retinal morphology, but this has not been addressed.
Results: To characterize the mechanism underlying light-induced blindness, we applied a molecular genetic approach using the fruit fly, *Drosophila melanogaster*. We found that the temporal loss of the photoresponse was paralleled by a gradual decline in the concentration of rhodopsin. The decline in rhodopsin and the visual response were suppressed by a C-terminal truncation of rhodopsin, by mutations in *arrestin*, and by elimination of a lysosomal protein, Sunglasses. Conversely, the visual impairment was greatly enhanced by mutation of the rhodopsin phosphatase, *rdgC*. Surprisingly, the mutations that suppressed light-induced blindness did not reduce the severity of the retinal degeneration resulting from constant light. Moreover, mutations known to suppress retinal degeneration did not ameliorate the light-induced blindness.
Conclusions: These data demonstrate that the constant light-induced blindness and retinal degeneration result from defects in distinct molecular pathways. Our results support a model in which visual impairment caused by continuous illumination occurs through an arrestin-dependent pathway that promotes degradation of rhodopsin.

Introduction

During the last 20 years, there has been considerable progress in understanding the genetic bases of retinal dystrophies. Of the 153 retinal disease loci that have been mapped, 103 of the genes have been identified [1] (http://www.sph.uth.tmc.edu/RetNet/). Environmental factors also contribute to retinal disease, and it has long been known that excessive exposure to light, even in wild-type animals, can be damaging to the retina and have deleterious effects on the photoresponse [2].

Considerable recent progress has been made in understanding the pathways underlying light-induced retinal degeneration, which can result from acute exposure of wild-type animals to very intense illumination or from long-term exposure to continuous low to moderate levels of light [2, 3]. These phenomena have been described in a large variety of vertebrate animal models ranging from zebrafish to rodents, rabbits, and monkeys [4–8]. Recently, evidence has shown that there are differences in the pathways that lead to bright- and low-light-induced retinal degeneration [9]. In mice, the bright-light-induced retinal degeneration occurs independently of transducin, the effector for rhodopsin, and requires the activity of the AP-1 transcription factor [9]. By contrast, signaling through transducin contributes to low-light-induced damage [9].

It has been presumed that photoresponse loss, resulting from excessive exposure to light, is merely a secondary consequence resulting from the severe morphological effects of damaging light. However, this has not been demonstrated. To examine further the mechanism by which low or moderate levels of continuous light may contribute to phototoxicity in wild-type animals, we used a genetic approach to explore whether the fruit fly is an effective model organism to study this process. We assayed the visual response by using a simple electrophysiological assay, electroretinogram (ERG) recordings, as a sensitive readout of phototoxicity. We found that constant-light-induced blindness occurred in flies. Unexpectedly, the mechanism that gave rise to this loss of the photoresponse was not a secondary consequence of retinal degeneration. Rather, the light-induced blindness in wild-type flies occurred through a molecular pathway distinct from that which caused retinal degeneration. Our results point to the importance of the rhodopsin degradation pathway in contributing to the blindness but not the retinal degeneration caused by continuous exposure to ambient light.

Results

Constant Light Impairs the Visual Response in *Drosophila*

To determine whether constant light leads to phototoxicity in *Drosophila*, we exposed wild-type flies to continuous, moderate levels of light and assayed the effects on the photoresponse by performing ERGs, which measure the summed responses of all retinal cells to light. In this analysis, we focused on the effects of light on the photoresponse because ERGs are more easily quantified than retinal degeneration and visual impairment is the key biological consequence resulting from phototoxicity. Young white-eyed (w^{1118}) flies maintained under a normal light/dark cycle displayed a corneal negative response to white or orange light; this response returned to the baseline after cessation of the light stimulus (Figure 1A and data not shown). Exposure of flies to blue light resulted in a prolonged depolarization afterpotential (PDA), due to stable activation of the major rhodop-

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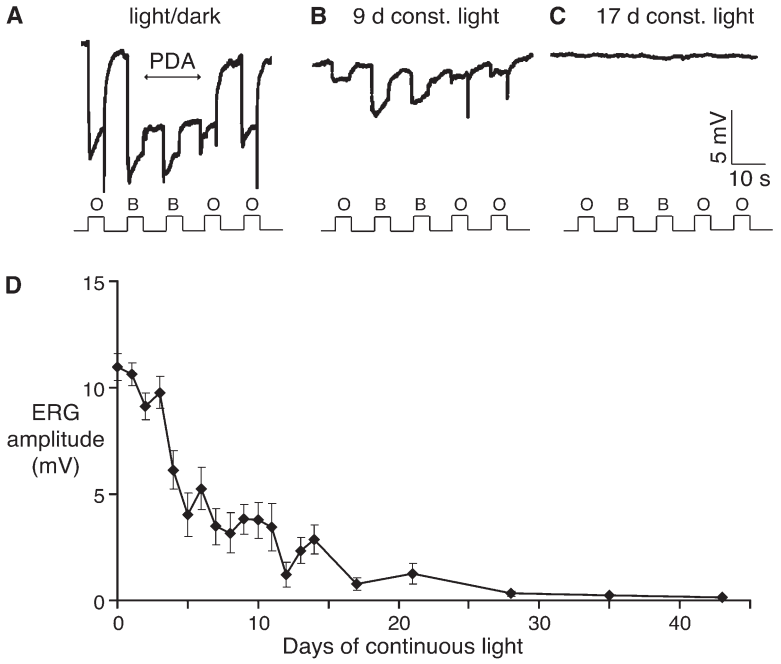

Figure 1. Continuous Light Results in Loss of the Visual Response in Wild-Type Flies

The ERGs were performed on young wild-type (w^{1118}) flies maintained during a 12 hr light/12 hr dark cycle (A) or after exposure to constant illumination from a white fluorescent light (30–40 mW/cm^2) for 9 days (B) or 17 days (C). To perform the ERGs, we stimulated the flies for 5 s each with either orange (O; 30 mW/cm^2) or blue (B; 5 mW/cm^2) light. Time and mV scales are shown in panel (C). (D) The average amplitudes of the ERGs from w^{1118} flies exposed to continuous light (30–40 mW/cm^2) for the indicated number of days. The error bars reflect the standard errors (n = 8–33).

sin, Rh1, even after cessation of the light stimulus (reviewed in [10, 11]). The PDA can be terminated by a short exposure to white or orange light, which facilitates the conversion of the activated metarhodopsin back to the nonactivated form of Rh1.

We found that wild-type flies maintained under constant illumination displayed a pronounced decrease in the photoresponse. After 9 days of continuous light at 18°C, the amplitude of the response was reduced, and most of the flies no longer displayed a PDA (Figures 1B and 1D). After 17 days of moderate light, the photoresponse was either eliminated or dramatically reduced (Figures 1C and 1D). The light-induced loss of the visual response was not due to a background mutation because similar impairments were observed in a variety of genetic backgrounds (Figure S1A in the Supplemental Data available with this article online). The diminished photoresponse must have been due to continuous light, rather than a strictly age-dependent effect, because the amplitudes of ERGs were not decreased in older flies exposed to 12 hr light/12 hr dark cyclic light (data not shown). In addition, there was no significant difference in sensitivity to phototoxicity between young (2- to 4-day-old) and old (3-week-old) wild-type flies maintained under constant light (Figure S1B). The gradual decline in the photoresponse was accompanied by retinal degeneration, as assessed in live flies by examination of the deep pseudopupil (DPP) [12] or by electron microscopy (Figures 2A and 2B). The DPP requires intact morphology, and even moderate degeneration causes the DPP to disappear. Thus, disappearance of the DPP is an indicator of the initiation of retinal degeneration; however, this assay does not permit discrimination between moderate and severe levels of retinal degeneration.

Ambient temperature has been shown to affect phototoxicity in mammals, and higher temperature can either exacerbate or ameliorate the effects of constant light [13, 14]. In *Drosophila*, we found that the time course of light-induced blindness was accelerated greatly at higher temperatures (Figure S2A). At 18°C, red-eyed flies did not show phototoxicity; however, at 29°C, the flies displayed light-induced blindness, although the process was slower than in w^{1118} flies (Figures S1C and S2B). High temperature alone did not cause visual impairment; the ERG amplitudes of w^{1118} flies reared for 7 days at 29°C in the dark were normal (Figure S2B).

The blindness induced by constant light was partially reversible. The small decrement in visual response resulting from exposure to constant light for three days was fully reversed after the flies were returned to the dark (Figure 3A). However, dark incubation only partially restored the loss in the ERG amplitude in flies exposed to constant light for 5 or more days (Figures 3A and 3B).

Constant-Light-Induced Reduction in Rhodopsin Concentration

The light-induced loss of the photoresponse raised the possibility that the concentration of one or more proteins critical for the photoresponse might be decreasing. The major rhodopsin, Rh1, was a particularly good candidate; we recently found that acute exposure of flies to very intense illumination for several hours results in rhodopsin degradation [15]. Therefore, we performed Western blots with head extracts obtained from flies that had been exposed to continuous ambient levels of illumination for 1–21 days. We found that the concentration of Rh1 gradually decreased after approximately 3 days of constant light (Figure 4A). By day 13, very little Rh1 was detected. In contrast to these results, the concentrations of other signaling proteins were largely unchanged after 7 days of continuous illumination and were still present at relatively high levels on day 17. These include the Gα, phospholipase C (PLC), the TRP

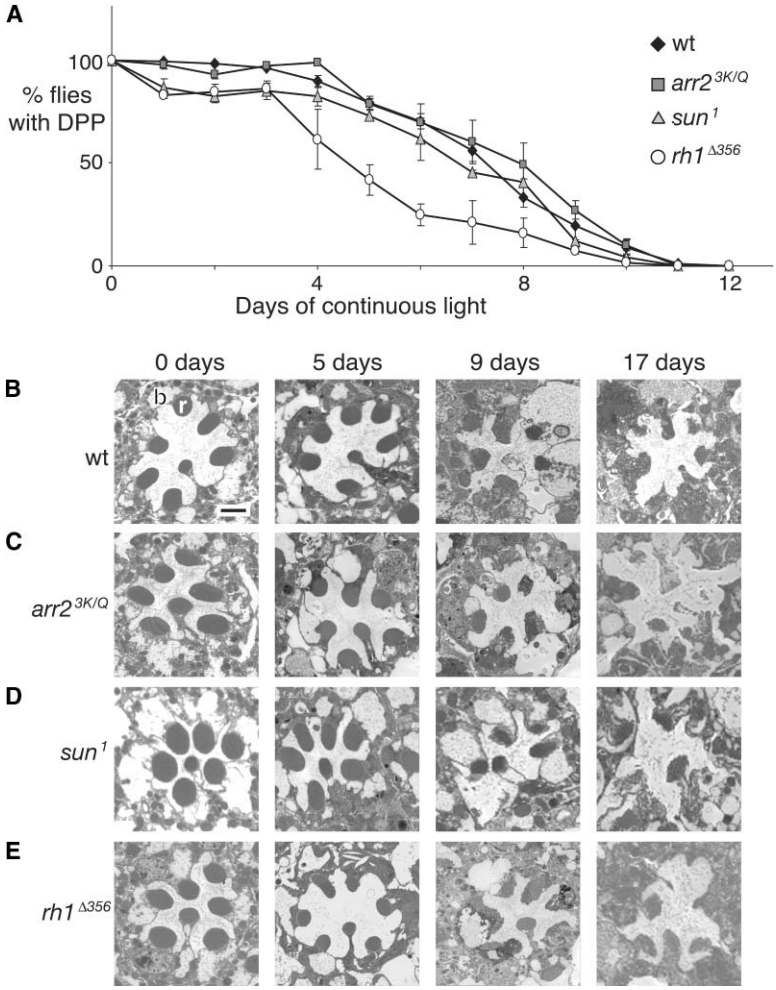

Figure 2. Continuous Light Results in Retinal Degeneration

(A) Counting the deep pseudopupil (DPP) allowed the time course of retinal degeneration to be assayed in live flies. A pattern of dark spots on the fly eye, the DPP results from the optical superposition of rhabdomeres from neighboring ommatidia [12]. The integrity of the DPP is correlated with the morphology of the ommatidia. Shown are the fraction of flies with DPPs as a function of days of continuous light (n = 30–50). The error bars represent the SEMs. (B–E) Transmission electron micrographs of cross-sections of compound eyes at a depth of 30 μm. Young flies (<2 days) were maintained under a normal light/dark cycle (0 day) or exposed to continuous light for 5, 9, or 17 days. (B) Wild-type (wt: w^{1118}). (C) *y w;;arr2*$^{3K/Q}$ (*arr2*$^{3K/Q}$). (D) w^{1118}*;;rh1*$^{\Delta 356}$ (*rh1*$^{\Delta 356}$). (E) w^{1118}*; sun*^{1} (*sun*^{1}). Abbreviations are as follows: r, rhabdomere; and b, cell body. The scale bar represents 2 μm.

cation channel, arrestin2, INAD, protein kinase C (PKC), and the minor rhodopsin (Rh4), which responds maximally to ultraviolet light (reviewed in [11, 16, 17]) (Figures 4B–4H). The levels of myosin III (NINAC) and the rhodopsin phosphatase (RDGC) decreased significantly after 17 days of continuous light (Figures 4I and 4J). However, in contrast to Rh1, the changes in the concentrations of NINAC and RDGC occurred after the loss of the ERG response. After 21 days of constant light, additional phototransduction proteins displayed significant reductions in protein concentrations (Figure 4). These results indicated that the light-induced loss of the photoresponse correlated with the reductions in Rh1 levels. The decreases in other signaling proteins lagged behind the effects of light on the photoresponse.

Suppression of Light-Induced Blindness by Mutations in *arrestin*, *sunglasses*, and *rh1*

To explore further the mechanisms by which continuous light leads to visual impairment, we asked whether mutations in candidate genes would enhance or suppress the decline in the ERG amplitude. We analyzed many mutations affecting phototransduction and retinal degeneration, but they had little if any suppressive effect (see below and data not shown). We then considered whether mutations in *arrestin2* (*arr2*) might suppress the light-induced blindness. Given that degradation of Rh1 may underlie the visual impairment, mutations that impact on Rh1 degradation could potentially alter the severity of light-induced blindness. We reasoned that Arr2 might contribute to the degradation of Rh1, but the likelihood of this proposal was difficult to predict because degradation of Rh1 during intense illumination is arrestin independent [15], whereas endocytosis of Rh1 in certain mutant backgrounds is arrestin dependent [18, 19].

To test whether the blindness induced by constant low or moderate light occurred through an arrestin pathway, we examined ERGs in *arr2* mutants. As described above, wild-type flies exposed to 9 days of constant light exhibited an approximately 3-fold reduction in the ERG amplitude relative to that of flies maintained in a light/dark cycle (Figures 1D and 5A). Elimination of Arr2 greatly suppressed the light-induced blindness; the amplitudes of the ERGs were similar in *arr2*^{5} null flies exposed to constant or cyclic light (Figure 5A). However, *arr2*^{5} displays significant retinal degeneration even in cyclic light [20], resulting in a small ERG amplitude under both sets of light conditions (Figure S4A). Therefore, we also analyzed the effects of an *arr2* allele, *arr2*$^{3K/Q}$, which is not characterized by pronounced reductions in the

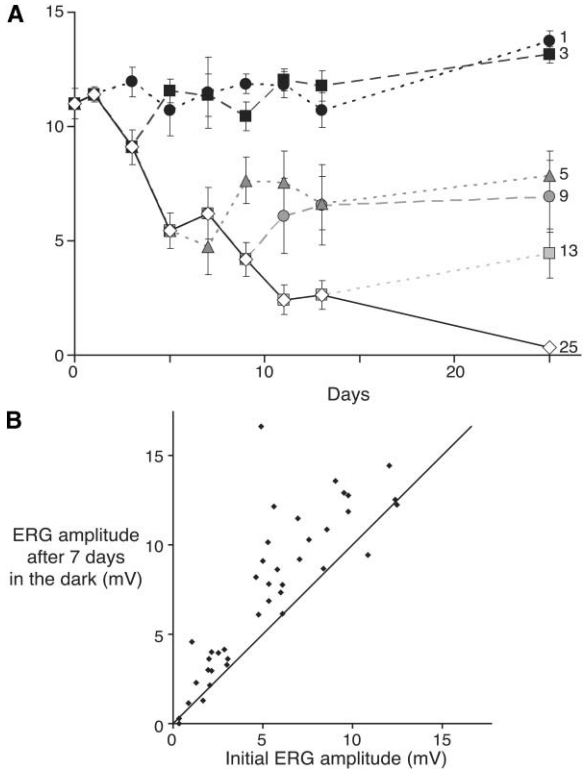

Figure 3. Partial Recovery of Light-Induced Visual Impairment in the Dark

(A) Partial recovery of ERG amplitudes assayed in populations of flies. Shown are the average ERG amplitudes from wild-type (*y w*^{67c}) flies exposed to continuous white light for 25 days (white diamonds connected by the solid black line). Subsets of flies were removed from constant light after the indicated periods and transferred to constant darkness: 1 day, black circles connected by short dashed black line; 3 days, black squares connected by long dashed black line; 5 days, gray triangles connected by short dashed gray line; 9 days, gray circles connected by long dashed gray line; or 13 days, gray squares connected by a short dashed gray line. The experiment was terminated after 25 days. The error bars represent the SEMs (n = 10–38).

(B) Performing ERGs on the same individual flies, immediately after exposure to constant light and again after incubation in the dark, tested for partial recovery from constant light. ERGs obtained at the same time point from the two eyes of the same flies were nearly identical (Figure S3). Here, the ERG amplitudes were obtained from the right eye of the flies (X axis) after 9 days of continuous illumination. The flies were then incubated in the dark for 7 days, and the ERGs were recorded from the left eyes of the same flies (Y axis). The line indicates Y = X. Longer incubations in the dark of up to 35 days did not result in additional improvement in the amplitudes of the ERGs (data not shown).

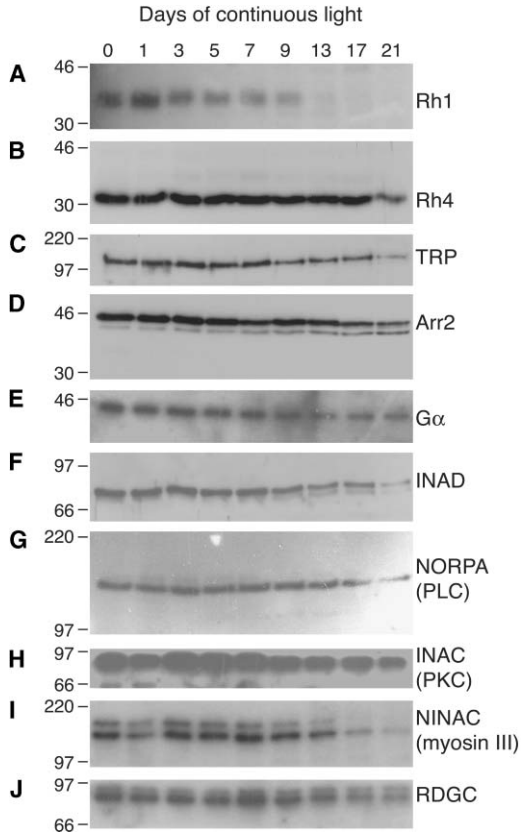

Figure 4. Concentrations of Signaling Proteins during Constant Light

Wild-type (*w*^{1118}) flies were exposed for 21 days to continuous illumination from a standard fluorescent light (30–40 mW/cm^2). Head extracts were collected after various days as indicated, and Western blots were probed with antibodies against various signaling proteins, which function in *Drosophila* visual transduction. (A) Rh1, (B) Rh4, (C) TRP, (D) Arr2, (E) Gα, (F) INAD, (G) NORPA (PLC), (H) INAC (PKC), (I) NINAC (myosin III), and (J) RDGC (rhodopsin phosphatase). The results were obtained from blots that were stripped and reprobed and/or from parallel blots containing the same set of samples.

ERG amplitude or retinal degeneration in cyclic light [21]. We found that the light-induced visual impairment was suppressed to a similar extent in *arr2*$^{3K/Q}$ and in null *arr2*^{5} flies exposed to continuous light for 9 days (Figure 5A). The suppression in *arr2*$^{3K/Q}$ flies was quite pronounced because these flies still had a significant ERG amplitude after 21 days of continuous light (Figure 5B). The light-induced decline in the ERG was also suppressed, although to a lesser extent, in *sunglasses*^{1} (*sun*^{1}) flies (Figures 5A and 5C), which fail to express a lysosomal tetraspanin that interacts with and promotes the degradation of Rh1 in lysosomes in response to acute exposure to bright light [15].

Particularly strong suppression was observed in a rhodopsin allele, *rh1*$^{\Delta 356}$, which expresses a version of Rh1 that removes most of the C-terminal cytoplasmic domain necessary for the formation of stable rhodopsin/arrestin complex [18, 22]. In *rh1*$^{\Delta 356}$ flies, the relative amplitude of the ERG was as high after 11 days of constant light as in newly eclosed flies (Figure 5D), although the initial amplitude was slightly smaller than in wild-type flies (Figure S4D).

A Mutation of Rhodopsin Phosphatase Enhances Light-Induced Visual Impairment

As with other G protein-coupled receptors, the C-terminal portion of Rh1 contains the major phosphorylation sites [22]. Dephosphorylation of Rh1 is mediated by a calmodulin-dependent protein phosphatase, Retinal Degeneration C (RDGC) [22–25], and in *rdgC* mutant flies there is an increased level of endocytosis of Rh1

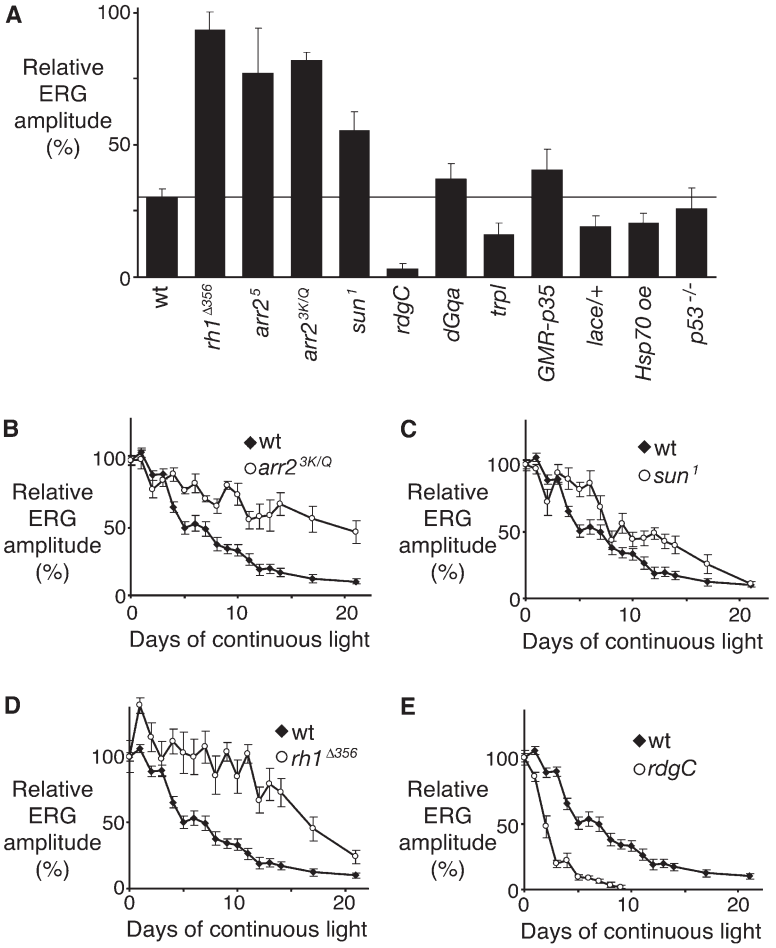

Figure 5. Genetic Suppression and Enhancement of Light-Induced Blindness

(A) Shown are the relative ERG amplitudes obtained from wild-type and mutant flies exposed to constant and cyclic light conditions. ERGs were recorded from the indicated fly stocks exposed to either 9 days of continuous white light (30–40 mW/cm^2) or 9 days of cyclic light (12 hr light/12 hr dark cycle). The average ERG amplitudes obtained from the wild-type or mutant flies maintained under cyclic lights were set as 100%. The relative ERGs were obtained by division of the average amplitudes obtained after constant light by the average amplitudes recorded after cyclic lights. The actual ERG amplitudes under both sets of conditions are presented in Figure S4 (in mV). The data for wild-type flies were obtained with *w*^{1118}, *y w*^{67c} and *cn bw* flies.

(B–D) Relative ERG amplitudes from wild-type (*w*^{1118}, *y w*^{67c} and *cn bw*) and the following mutant stocks exposed to constant light: (B) *y w;;arr2*$^{3K/Q}$ (*arr2*$^{3K/Q}$); (C) *w*^{1118}*;sun*^{1} (*sun*^{1}); (D), *w*^{1118}*;;rh1*$^{\Delta 356}$ (*rh1*$^{\Delta 356}$); and (E) *y w*^{67c}*;;rdgC*^{306} (*rdgC*). The average ERG amplitudes from the corresponding flies at day 0 were defined as 100%. Each data point represents the mean ERG values from ≥8 flies. The standard errors are indicated. The actual ERG amplitudes (in mV) used to obtain the relative ERGs (for panels [B–E]) are shown in Figure S4.

[19]. Thus, *rdgC* could potentially have the opposite effect on light-induced blindness as *rh1*$^{\Delta 356}$. Consistent with this proposal, we found light-induced blindness was greatly accelerated in *rdgC* flies (Figures 5A and 5E).

Distinct Effects of Constant Light on the Visual Response and Retinal Morphology

Surprisingly, despite the suppression of light-induced blindness by the *arr2*$^{3K/Q}$ and *sun*^{1} mutations, the rhabdomeres in these mutant flies exhibited degeneration as strong as that in wild-type flies similarly treated with constant light (Figures 2A–2D). In fact, when we used the DPP assay, which is very sensitive to perturbations in rhabdomere morphology, the initiation of retinal degeneration appeared to occur earlier in *rh1*$^{\Delta 356}$ flies than in wild-type flies (Figures 2A, 2B, and 2E). The failure of constant light to rescue retinal degeneration in these mutants was unexpected given that *arr2*$^{3K/Q}$ and *rh1*$^{\Delta 356}$ mutations suppress a variety of genetically induced retinal degenerations [21, 22]. These data suggest that the mechanisms leading to light-induced visual impairment and the structural light-dependent retinal degeneration might be distinct.

In contrast to the effects of the *rh1*$^{\Delta 356}$, *arr2*$^{3K/Q}$, and *sun* mutations, the severity of light-induced blindness was not suppressed by mutations or overexpression of gene products shown to suppress genetic forms of retinal degeneration in flies or the retinal degeneration caused by excessive light in mammals. We found that there was no significant effect resulting from either overexpression of the antiapoptotic baculovirus p35 protein [26] or elimination of one copy of a serine C-palmitoyltransferase (*lace*/+) [27] (Figure 5A), which suppresses some genetically induced retinal degenerations in *Drosophila*. Increased expression of *hsp70* in photoreceptor cells also did not reduce visual impairment (Figure 5A), although increased levels of this chaperone prevented neuronal death in a fly model for Parkinson's disease [28]. A mutation that knocked out the proapoptotic gene *Drosophila* p53, which has been shown to suppress cell death in the developing retina in response to ultraviolet light [29], also had no effect on the severity of constant light-induced blindness (Figure 5A). In the mouse, retinal degeneration induced by constant low light is suppressed by a mutation in the effector for the rhodopsin transducin [9]. However, a hypomorphic mutation in the effector for Rh1, $G\alpha_q$, did not impact significantly on the severity of the light-induced visual impairment (Figure 5A), although it did decrease the amplitude of the ERG [30, 31] (Figure S4A). The combination of these results is consistent with the conclusion that the light-induced blindness occurs through a mechanism distinct from that of many forms of retinal degeneration.

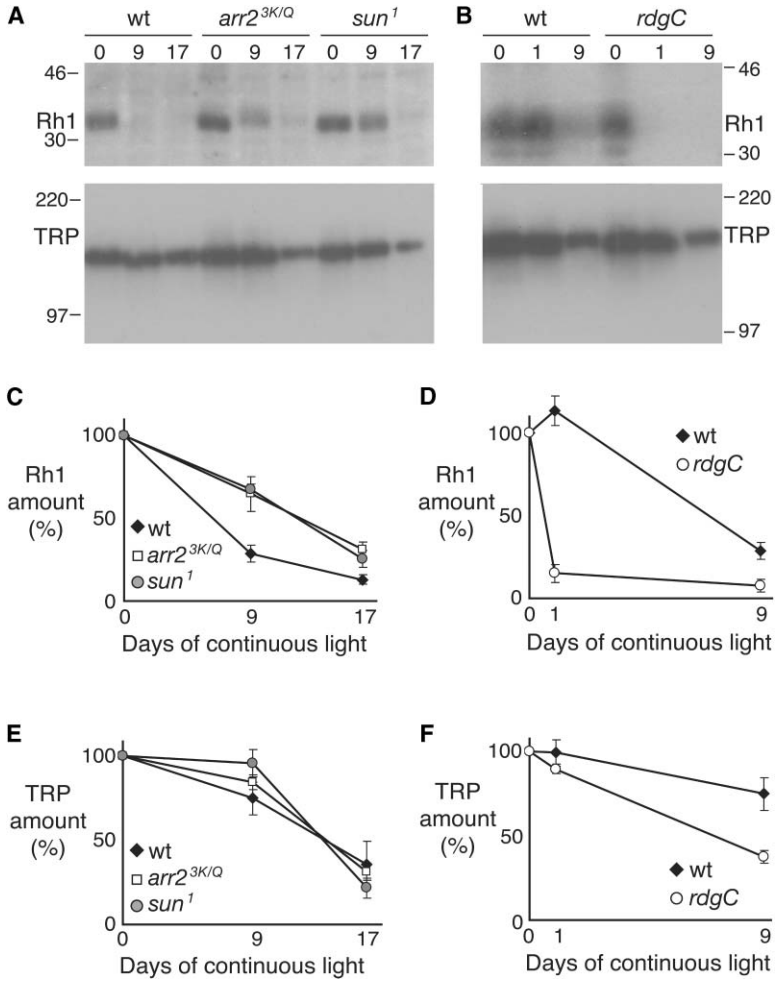

Figure 6. Genetic Control of Light-Dependent Levels of Rhodopsin (Rh1)

(A) Suppression of light-dependent reduction in Rh1 levels in *arr2*$^{3K/Q}$ and *sun*^{1}. The following fly stocks were maintained under a light/dark cycle (0 days) and then exposed to continuous illumination from a standard fluorescent light (30–40 mW/cm^2) for 9 or 17 days: (1) wild-type (wt: *w*^{1118}), (2) *y w;;arr2*$^{3K/Q}$ (*arr2*$^{3K/Q}$), and (3) *w*^{1118}*;sun*^{1} flies (*sun*^{1}). The Western blot containing head extracts was probed with anti-Rh1 antibodies. A parallel Western blot, with the same samples, was probed with anti-TRP antibodies.

(B) Light-dependent reduction in Rh1 levels in *rdgC* flies. Newly eclosed wild-type (*w*^{1118}) and *y w*^{67c}*;;rdgC* flies (*rdgC*), which were reared in the dark (0 day), were exposed to continuous light for 1 or 9 days. Parallel Western blots were probed with anti-Rh1 and anti-TRP antibodies. A phosphoimager was used for quantification of Rh1 (C and D) and TRP (E and F) levels from panels (A) and (B). Error bars indicate the SEMs (n = 3–6).

Genetic Enhancement and Suppression of Light-Induced Degradation of Rh1

The preceding data raise the possibility that the enhancement and suppression of the visual impairment resulting from constant light may be due to effects on degradation of Rh1. To test this, we maintained *arr2*$^{3K/Q}$, *sun*^{1}, and *rdgC* flies in constant light and examined the time-dependent changes in the level of Rh1. We could not assess the level of Rh1 in *rh1*$^{\Delta 356}$ flies because the anti-Rh1 antibodies did not recognize the truncated Rh1$^{\Delta 356}$ protein (Figure 7A, lower panel). We found that the large decrease observed in Rh1 concentration in wild-type flies was suppressed in both *arr2*$^{3K/Q}$ and *sun*^{1} to similar extents (Figures 6A and 6C). The greater suppression by the *sun*^{1} mutation on Rh1 degradation than on light-induced visual impairment may be due to the accumulation of nonfunctional Rh1 in a large array of loops extending into the cell bodies of *sun*^{1} photoreceptor cells [15]. Other critical phototransduction proteins may not be present in these invaginations, thereby rendering this portion of the Rh1 pool unable to contribute effectively to the photoresponse.

In contrast to the suppression of light-induced Rh1 degradation in *arr2* and *sun*^{1} mutant flies, the levels of Rh1 decreased much more rapidly in *rdgC* relative to wild-type flies (Figures 6B and 6D). Although in wild-type animals there was no decrease in Rh1 after 1 day of continuous illumination, very little Rh1 was detected in identically treated *rdgC* flies. Consistent with this finding, the PDA, which depends on high levels of Rh1 levels, was eliminated in *rdgC* flies exposed to constant light for 1 day (Figure S5).

Continuous Light Increases Rh1/Arr2 Complexes in Wild-Type Flies

It has been shown previously that mutations in several genes required for phototransduction result in stable Rh1/Arr2 complexes and lead to an increase in endocytosis of Rh1 [18, 19, 32]. Because endocytosis is a prerequisite for downregulation of G protein coupled receptors [33], we considered whether constant light led to increased formation of stable Rh1/Arr2 complexes in wild-type flies during the early stages of light-induced phototoxicity.

To assay the levels of Rh1/Arr2 complexes in wild-type flies exposed to constant light, we performed a variation of an arrestin pelleting assay [20]. Previous analyses of Rh1/Arr2 complexes compared the levels of bound Arr2 after either a short pulse of blue light, which promotes the production of stable metarhodopsin

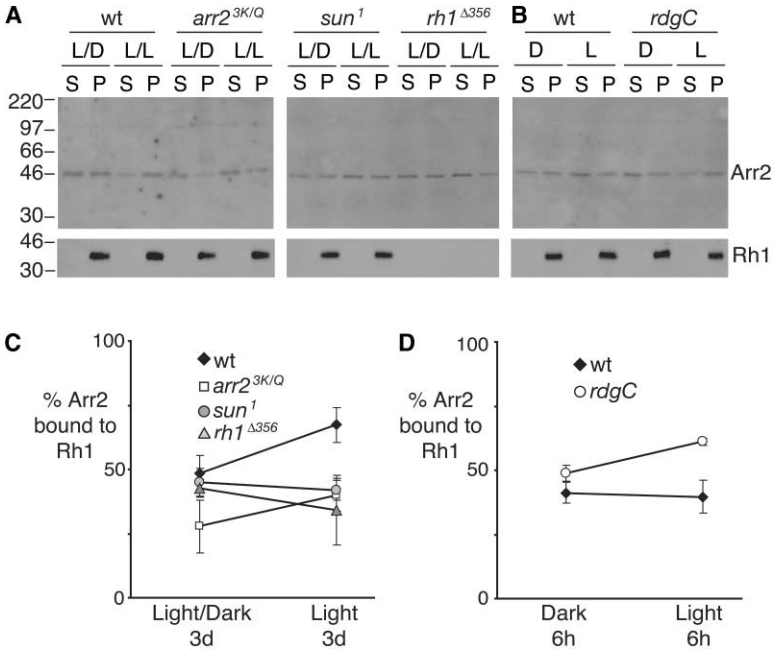

Figure 7. Genetic Manipulation of Stable Constant Light-Induced Rhodopsin/Arrestin Complexes

The relative proportions of Arr2 in the supernatant and pellet fractions were determined. Arr2 bound to Rh1 fractions in the pellet because Rh1 is very hydrophobic and remains in the pellet, whereas the unbound Arr2 is in the supernatant. The supernatant (S) and pellet (P) fractions were collected under ambient light and fractionated by SDS-PAGE. Western blots were probed with anti-Arr2 and anti-Rh1 antibodies. (A) The following fly stocks were maintained under a light/dark cycle for 3 days (L/D) or illuminated constantly for 3 days (L/L): (1) wild-type (wt: w^{1118}), (2) $y\ w;;arr2^{3K/Q}$ ($arr2^{3K/Q}$), (3) $w^{1118};sun^{1}$ (sun^{1}), and (4) $w^{1118};;\ rh1^{\Delta356}$ ($rh1^{\Delta356}$). (B) Newly eclosed wild-type (w^{1118}) and $y\ w^{67c};;rdgC^{306}$ flies (*rdgC*) were either incubated in the dark or exposed to continuous light for 6 hr. (C and D) The proportion of Arr2 bound to rhodopsin in A and B, respectively. The percent of bound Arr2 was determined with a phosphoimager. The error bars represent the SEMs (n = 3–4).

and association of Arr2, or after a subsequent pulse of orange light, which results in the back conversion to rhodopsin and dissociation of Arr2. To determine whether constant light increased the level of stable Rh1/Arr2 complexes, we compared the level of bound Arr2 in flies maintained under cyclic lights or continuous low levels of white light for 3 days. We found that wild-type flies exposed to 3 days of constant light exhibited an increased concentration of stable Rh1/Arr2 complexes when they were compared to flies maintained under a light/dark cycle for the same duration (Figures 7A and 7C).

If the increase in Rh1/Arr2 complexes underlies the constant light-induced degradation of Rh1, then the concentration of these complexes should be decreased by mutations that suppress Rh1 degradation and phototoxicity. Consistent with this proposal, we found that the levels of stable Rh1/Arr2 complexes were similar in sun^{1}, $rh1^{\Delta356}$, and $arr2^{K/Q}$ flies, which were exposed to continuous light, and wild-type flies incubated under a light/dark cycle (Figures 7A and 7C). Stable Rh1/Arr2 complexes were also lower in $arr2^{3K/Q}$ flies exposed to cyclic light than similarly treated wild-type flies.

We next tested whether there was a greater concentration of stable Rh1/Arr2 complexes in *rdgC* flies than in wild-type flies. Because the level of Rh1 significantly decreased even after 1 day of constant light (Figures 6B and 6D), we used newly eclosed flies treated for only 6 hr with constant low light or held in the dark. Arrestin associates with rhodopsin upon light stimulation and dissociates from rhodopsin in the dark, even after a dark incubation as short as several seconds. Therefore, to compare differences in the relative concentrations of stable rather than transient Rh1/Arr2 complexes, we briefly exposed both light- and dark-treated flies with ambient white light immediately before the sample preparation. In wild-type flies, 6 hr of light had no impact on the concentration of Rh1/Arr2 complexes (Figures 7B and 7D). However, in *rdgC* flies, 6 hr of illumination caused an increase in the amount of arrestin bound to Rh1 (Figures 7B and 7D). Thus, the light-dependent changes in Rh1 concentration and Rh1/Arr2 complexes were suppressed in $arr2^{3K/Q}$ and sun^{1} and enhanced in *rdgC* flies.

Discussion

Distinct Mechanisms Underlie Light-Induced Blindness and Retinal Degeneration

It is a longstanding observation that exposure of wild-type animals to constant light leads to retinal degeneration [2]. In the current work, we set out to determine if constant light caused phototoxicity in *Drosophila* and, if so, whether we could characterize the mechanism further by using a genetic approach. As a sensitive and quantitative assay for phototoxicity, we focused on the effects of continuous light on the photoresponse by performing ERGs. We found that flies maintained under continuous ambient light for many days gradually lost their visual response and eventually went blind.

An important unanticipated finding was that the mechanism of light-induced blindness was distinct from that underlying light-induced retinal degeneration. We found that mutations such as *arrestin2* and *sun* that suppressed the light-induced blindness did not suppress the retinal degeneration resulting from exposure to the identical light conditions. In fact, a C-terminal deletion of rhodopsin ($Rh1^{\Delta356}$), which significantly reduced the severity of visual impairment by continuous illumination, actually accelerated the morphological damage resulting from constant ambient light. The lack of suppression of the low-light-induced retinal degeneration in wild-type flies was striking because the $rh1^{\Delta356}$ and the $arr2^{3K/Q}$ alleles analyzed here greatly suppressed certain genetically induced retinal degenerations [18, 21, 22].

The results in the current study demonstrate that the

loss of the photoresponse due to continuous light is not simply a secondary consequence of the retinal degeneration, which occurs in parallel with the visual impairment. Rather, light-induced apoptosis and blindness result from perturbations in different processes. This point is further illustrated by the findings that mutations in or overexpression of proteins known to suppress apoptosis in flies and other organisms do not ameliorate the light-induced blindness. Furthermore, although disruption of *arrestin2* suppressed the visual defects caused by continuous illumination, mutations in arrestin actually cause retinal degeneration in the presence of cyclic light in both flies and the mouse [34, 35].

Mechanism Underlying Blindness Induced by Continuous Light in Wild-Type Animals

The combination of results presented here indicates that the low-light-induced blindness was due to a decline in rhodopsin levels. An indication that this was the case was that Rh1 was the only protein that declined in parallel with the visual impairment. Furthermore, mutations that either decreased or increased the severity of the Rh1 degradation caused a comparable suppression or enhancement of the visual impairment. However, genetic suppression of the light-induced decline in Rh1 levels did not reduce the retinal degeneration resulting from constant light. Thus, the decrease in the concentration of rhodopsin did not appear to underlie the retinal degeneration.

A key question concerns the mechanism through which continuous low light caused a large reduction in Rh1 levels. We have recently shown that the Rh1 degradation resulting from acute exposure to very bright light occurred through an arrestin-independent pathway, which remains to be defined [15]. In contrast to these results, mutations in *arrestin2* suppressed the low-light-induced loss of Rh1. The differences in the mechanisms underlying bright- versus low-light-induced blindness are somewhat reminiscent of a recent mouse study demonstrating that retinal degeneration caused by extended exposure to low light is caused by a different mechanism than that for retinal degeneration caused by brief exposure to very bright light [9]. However, the similarities between this recent report and the current study are limited because the light-induced loss of the ERG is not due to retinal degeneration. Although elimination of the trimeric G protein suppressed the retinal degeneration in the mouse, a hypomorphic allele of the $G\alpha_q$ did not reduce the severity of low-light-induced blindness in the fly, although the amplitude of the ERG was reduced as a result of a decreased concentration of the G protein.

Arrestin was originally characterized as a regulatory protein that functions in the inactivation of rhodopsin and other G protein-coupled receptors (GPCRs) (reviewed in [33]). More recently, arrestin has been shown to bind clathrin and, under some conditions, to participate in endocytosis of GPCRs (reviewed in [33, 36]). The interaction between rhodopsin and arrestin is usually transient and typically does not lead to endocytosis of rhodopsin. However, in mutant backgrounds that remove the rhodopsin phosphatase (RDGC) or phospholipase C (NORPA), which is the effector for the G protein, the *Drosophila* rhodopsin Rh1 is stably bound to arrestin, leading to endocytosis [18, 19, 32]. Once internalized through endocytosis, GPCRs are either recycled to the plasma membrane or degraded (reviewed in [33]). In the case of the *norpA* and *rdgC* mutant flies, it is not known if the internalized Rh1 is ultimately recycled or degraded. Moreover, stable rhodopsin/arrestin complexes had not previously been observed in wild-type photoreceptor cells.

The results in this work support a molecular model in which constant light leads to blindness through a multistep process initiated by the formation of stable rhodopsin/arrestin complexes and culminating with the loss of the light receptor, Rh1. We found that a continuous low or moderate level of illumination, in the absence of any mutation, promotes the formation of stable rhodopsin/arrestin complexes. The concentration of Rh1 gradually declined, through a process involving the photoreceptor cell enriched lysosomal protein Sunglasses [15].

The preceding pathway underlying low-light-induced blindness was supported by genetic evidence. It has been shown previously that deletion of the C terminus of Rh1 prevents the formation of stable rhodopsin/arrestin complexes, which result from certain genetic perturbations that dramatically disrupt phototransduction [18, 19]. We found that, in wild-type flies exposed to constant illumination, the truncated Rh1 ($Rh1^{\Delta 356}$) also interfered with the formation of Rh1/Arr2 complexes and greatly suppressed light-induced blindness. In addition, *arrestin2* mutations suppressed the light-induced decline in Rh1 and the impairment in the photoresponse. Elimination of the photoreceptor cell enriched lysosomal protein Sun [15] also reduced the severity of the light-induced blindness, but to a lesser extent than in $rh1^{\Delta 356}$ or $arr2^{3K/Q}$ mutant backgrounds.

Our data suggest that the formation of rhodopsin/arrestin complexes is the key step determining the extent of Rh1 degradation and visual impairment in response to constant light. Additional evidence in support of this model is that the harmful effect of continuous light on the photoresponse is accelerated significantly in a genetic background, *rdgC*, which increases Rh1/arrestin complexes and Rh1 degradation.

Implications for Mammalian Light-Induced Blindness

We propose that mammalian visual impairment, which results from exposure to continuous but low-intensity light, may also occur through an arrestin-dependent mechanism and reductions in rhodopsin levels. According to this model, stable rhodopsin/arrestin complexes and endocytosis/degradation of rhodopsin do not normally occur to any significant extent in wild-type animals. Rather, as a result of continuous light, the rhodopsin concentration gradually decreases through an arrestin-dependent pathway. It will be interesting to determine whether mutations that affect arrestin trafficking in mammals also suppress visual impairment resulting from constant light.

The model presented here differs from the "equivalent-light hypothesis," which proposes that phototoxic-

ity and retinal degeneration resulting from continuous light are due to constitutive activation of signaling by rhodopsin or other phototransduction molecules [37]. Although there is compelling evidence that the equivalent-light hypothesis applies to certain forms of morphological degeneration in the retina, our data indicate that the light-induced blindness occurs through an increase in stable rhodopsin/arrestin complexes and degradation of rhodopsin. This conclusion is also supported by our observation that the hypomorphic allele of the $G\alpha_q$ does not suppress the visual impairment in flies. Although it remains to be determined whether rhodopsin/arrestin complexes occur in wild-type mammals in response to continuous low light, it has been shown that intense levels of light cause a decline in rhodopsin levels in vertebrates (reviewed in [38]). It will be interesting to address whether high- and low-light-induced degradation of mammalian rhodopsin occur through arrrestin-independent and arrestin-dependent mechanisms, respectively, as is the case in *Drosophila*.

Concluding Remarks

A relevant question is why a mechanism exists for formation of stable rhodopsin/arrestin complexes and degradation of rhodopsin if this phenomenon has negative consequences for the visual response. As previously suggested [18], endocytosis and degradation of stable rhodopsin/arrestin complexes may normally occur at very low levels and provide a quality control mechanism for eliminating photodamaged rhodopsins, which might otherwise accumulate in photoreceptor cells and have deleterious effects. Thus, the constant-low-light-induced blindness in wild-type animals would appear to be a pathological consequence resulting from excessive activity of a quality control mechanism, which is normally protective.

Experimental Procedures

Fly Stocks

The experiments were performed with the following white-eyed (*w*) strains (≤2 days posteclosion except as otherwise noticed) reared at 25°C under a 12 hr light/12 hr dark cycle: (1) w^{1118}, (2) $y\ w^{67c}$, (3) *cn bw*, (4) Canton S, (5) ry^{506}, (6) $w;;ninaE^{\Delta 356}$ ($rh1^{\Delta 356}$) [22], (7) $w;;arr2^{5}$ [20], (8) $y\ w;;arr2^{5}$ P[$y+$, $arr2^{3K/Q}$] ($arr2^{3K/Q}$) [21], (9) $w;sun^{1}$ [15], 10) $y\ w^{67c};;rdgC^{306}$ (*rdgC*) [23], (11) *w;dGqα* [31], (12) $w;trpl^{302}$ [39], (13) *w;;GMR-p35* [26], (14) $w;lace^{2}/+$, 15) *cn bw;GMR-Gal4/UAS-hsp70* (*hsp70 oe*) [28], (16) *y w;;p53* [*5A-1-4*], and 17) *y w;;p53* [*11-1B-1*] [40].

Continuous Light Treatment

The flies were placed in an incubator set at 18°C, 25°C, or 29°C. Two fluorescence bulbs (BIAX F13BX/SPX27) were used as the light sources to minimize the generation of heat. The distance from the light sources to the flies was 8 cm to the bottom of the cotton plug and 12 cm to the top of the food. The light intensities to which the flies were exposed ranged from 30 to 40 mW/cm depending on their locations within the vials. The vials were changed every 3–4 days at 18°C, every 2 days at 25°C, and everyday at 29°C so that the environment remained as constant as possible. The vial locations were changed randomly everyday so that the possible effects resulting from small deviations in light intensities within the incubators would be minimized.

Electroretinograms

The ERGs were performed as described [21] with a series of orange (580 nm; 30 mW/cm^2) and blue (480 nm; 5 mW/cm^2) light stimuli.

Light-Induced Changes in Rh1 Concentration

Light-induced changes in Rh1 levels were analyzed for the following stocks: (1) w^{1118}, (2) $w;sun^{1}$, (3) $w;;arr2^{3K/Q}$, and (4) $y\ w;;rdgC^{306}$. The flies were reared in the dark and then exposed to either a normal light/dark cycle or continuous white fluorescent light (30–40 mW/cm^2) for the indicated durations (see figure legends). Ten fly heads were homogenized in 100 μl of Tris-buffered saline [20 mM Tris (pH 7.5), 150 mM NaCl] containing 1% Triton X-100 and protease inhibitors and then mixed with 100 μl of 2× SDS sample buffer. The extracts were fractionated by SDS-PAGE (12%), and the Western blots were probed with mouse anti-Rh1 antibodies [41] followed by ^{125}I-labeled anti-mouse antibodies (NEN). Separate blots containing the same extracts were probed with the following antibodies generated in rabbits: anti-TRP [42], anti-Arr2 (gift from S. Subramanium), anti-Rh4 (gift from S. Subramanium), Gα (T. Wang and C.M., unpublished data), NORPA (PLC) (T. Wang and C.M., unpublished data), INAC (PKC) [43], NINAC [44], RDGC [25], and INAD [45]. The signals were detected with either an enhanced chemiluminescence (ECL) kit (Perkin Elmer) or ^{125}I-labeled protein A (ICN). For the quantification of the protein concentrations in Figure 6, the membranes were exposed to a BAS-III imaging plate (Fuji Film) with a phosphoimager (BAS-1500, Fuji Film).

Arrestin Pelleting Assay

Arr2 binding assays were performed as described [20] with minor modifications. The flies were exposed to a normal light/dark cycle, to continuous white fluorescent light (30-40 mW/cm^2), or to constant darkness for the indicated durations (see figure legends). Ten fly heads were dissected and added to Tris-buffered saline containing 5 mM DTT and protease inhibitors, homogenized under ambient light conditions, and centrifuged at 14,000 rpm for 5 min. Pellet and supernatant fractions were separated and subjected to SDS/PAGE and Western analysis with anti-Arr2 and anti-Rh1 antibodies. Quantification of the levels of Arr2 was performed with ^{125}I-labeled protein A and a phosphoimager.

Transmission Electron Microscopy

Transmission EM was performed as described [21]. In brief, fly heads were hemisected, fixed in a buffered paraformaldehyde and glutaraldehyde solution, and embedded in LR White resin, and the tangential sections were cut at a depth of 35 μm from the surface of the eyes.

Supplemental Data

Supplemental data are presented in five figures available with this article online at http://www.current-biology.com/cgi/content/full/14/23/2076/DC1/.

Acknowledgments

We thank Mike Sepanski for preparing sections of compound eyes, Dr. S. Subramanium for anti-Arr2 and anti-Rh4 antibodies, Dr. H. Xu for help with preparing the figures, and Dr. R. E. Anderson for consultation. This work was supported by a grant to C.M. from the National Eye Institute (EY08117).

Received: October 11, 2004
Revised: October 12, 2004
Accepted: October 12, 2004
Published: December 14, 2004

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